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(54)【発明の名称】　生体成分のアフィニティ分析法

(57)【要約】

【目的】　測定対象物質と標識を含む免疫複合体を、固相より迅速、容易に、かつ選択的に乖離させ、非特異的吸着画分由来のバックグランド低減を可能にした高感度分析法を提供する。

【構成】　測定対象物に対して親和性を有する物質に核酸を結合した核酸結合体と、該核酸と相補的な核酸を結合させた固相とを用い、測定対象物と該核酸結合体と酵素とを含む免疫複合体を、前記固相に結合された核酸と該核酸結合体とをその相補結合によって不動化した状態に形成させた後、該相補結合の安定性に関与する因子を調節することによって測定対象物と酵素を含む免疫複合体を該固相から選択的に乖離させる。

【特許請求の範囲】

【請求項1】　生体試料中の測定対象物を固相上に選択的に捕捉して該試料中の共存物質と分離した後に測定する分析法であって、

測定対象物に対して親和性を有する物質に第1の核酸を結合させた核酸結合体と、第1の核酸と相補的に結合し得る第2の核酸を固相に結合させた核酸固定固相とを用い、これら第1の核酸と第2の核酸が相補結合する条件下で前記核酸結合体に含まれる測定対象物に対して親和性を有する物質と前記試料中の測定対象物とを反応させて、少なくとも測定対象物質と第1の核酸を含んだ複合体を前記核酸の相補結合を介して固相上に不動化させ、

固相を試料と分離した後、第1の核酸と第2の核酸の相補結合が解離する条件下で測定対象物を含む複合体を固相から遊離させて固相を除いた測定液を得ることを特徴とする生体成分のアフィニティ分析法。

【請求項2】　生体試料中の測定対象物を固相上に選択的に捕捉して該試料中の共存物質と分離した後に測定する方法であって、

測定対象物に対して親和性を有する物質に第1の核酸を結合させた核酸結合体と、第1の核酸と相補的に結合し得る第2の核酸を固相に結合させた核酸固定固相とを用い、前記核酸結合体と前記試料中の測定対象物とを反応させて少なくとも測定対象物と第1の核酸を含んだ複合体を形成させ、該複合体中の第1の核酸を前記第2の核酸と相補結合させて少なくとも測定対象物と第1の核酸を含んだ複合体を前記核酸の相補結合を介して固相上に不動化させ、

固相を試料と分離した後、第1の核酸と第2の核酸の相補結合が解離する条件下で測定対象物を含む複合体を固相から乖離させて固相を除いた測定液を得ることを特徴とする生体成分のアフィニティ分析法。

【請求項3】　第1の核酸と第2の核酸の相補結合を解離する条件を、該相補結合の安定性に関与する因子である塩濃度又は温度の少なくともいずれかの制御により与えることを特徴とする請求項1又は2に記載のアフィニティ分析法。

【請求項4】　請求項1ないし3のいずれかにおいて、測定対象物質と第1の核酸を含む複合体は、測定用の標識物質を有していることを特徴とする生体成分のアフィニティ分析法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、生体成分をそれらが有する相互作用を利用して分析する方法に関する。

【0002】

【従来の技術】生体試料中の微量成分を特異的かつ高感度に測定する方法として、測定対象成分に対する抗体を用いたイムノアッセイや受容体を用いたレセプターアッセイなどがある。

【0003】高感度イムノアッセイの場合の測定原理を以下に示すと、これは、まず測定対象成分（抗原）に対する抗体をチューブ、ビーズ、プレートなどの担体に固定化（固相化）させておき、これに試料を添加して抗原を固相化抗体に捕捉させる。試料中の共存物質を除去した後、抗原に対して固相化抗体とは認識部位の異なる抗体の標識抗体（抗体に標識物を結合させた抗体）を過剰に添加し、固相上に捕捉された抗原に結合させる。前記標識抗体の標識物としては、放射性同位元素、酵素、蛍光物質、発光物質などが用いられる。結合した標識抗体は抗原量に比例するため、未反応の標識抗体を洗浄除去した後、固相上の標識物を定量することにより抗原量を求める。

【0004】しかし前記の測定原理においては、標識抗体を過剰に添加するため、固相表面に非特異的に吸着した標識抗体が完全に洗浄除去されずに残り、測定系のバックグランドを上げる原因となって高感度化が達成されないという問題がある。

【0005】すなわち、前述の如き従来方法では、固相の担体を含む反応液に過剰の標識抗体を添加し、洗浄した後、抗原－抗体反応によって固相上に形成されている複合体の標識物を該固相と分離することなく直接検出するものであるため、固相表面に非特異的に吸着している標識抗体も同時に検出される結果となり、これがバックグランド上昇を招く結果となっていた。

【0006】そこで、固相上に形成させた免疫複合体を固相から切断した後、標識物を定量する試みがなされた例があり、例えば、免疫複合体をジスルフィド結合を介してゲルに捕捉させた後、還元剤を用いて結合を切断し、免疫複合体を溶出させ標識物を定量する方法が報告されている（臨床検査，２８，９０９～９１６，１９８４）。しかしこの方法では、固相からの切断に用いる還元剤が抗体分子を変性させ、非特異吸着している標識抗体が脱離したり標識物が乖離したりして、測定対象となる免疫複合体を選択的に切断させることができなかった。

【0007】また免疫複合体を核酸を介して固相に捕捉させた後、制限酵素を用いて該核酸を切断し、標識物を定量する方法が報告されている（今井他、特開平４－２０４３７９号）。しかしこの方法では、酵素反応を利用した切り出し法であるため、その工程に長時間を要し、全体の測定時間の短縮化、低ランニングコスト化を図る上において不利である。

【0008】

【発明が解決しようとする課題】本発明の目的は、測定対象物質と標識を含む免疫複合体を、固相より迅速、かつ選択的に乖離させることができ、これにより、固相に対して非特異的に吸着する画分由来のバックグランド上昇の影響を低減可能とした高感度分析法を提供することにある。

【0009】また本発明の別の目的は、固相から測定対象物を選択的に乖離させる方法として、制御が容易な方法を提供するところにある。

【0010】

【課題を解決するための手段】すなわち前記した請求項1の発明は、生体試料中の測定対象物を固相上に選択的に捕捉して該試料中の共存物質と分離した後に測定する分析法であって、測定対象物に対して親和性を有する物質に第1の核酸を結合させた核酸結合体と、第1の核酸と相補的に結合し得る第2の核酸を固相に結合させた核酸固定固相とを用い、これら第1の核酸と第2の核酸が相補結合する条件下で前記核酸結合体に含まれる測定対象物に対して親和性を有する物質と前記試料中の測定対象物とを反応させて、少なくとも測定対象物質と第1の核酸を含んだ複合体を前記核酸の相補結合を介して固相上に不動化させ、固相を試料と分離した後、第1の核酸と第2の核酸の相補結合を解離する条件下で測定対象物を含む複合体を固相から遊離させて固相を除いた測定液を得ることを特徴とする生体成分のアフィニティ分析法を提供するものである。

【0011】また前記請求項2の発明の特徴は、生体試料中の測定対象物を固相上に選択的に捕捉して該試料中の共存物質と分離した後に測定する方法であって、測定対象物質に対して親和性を有する物質に第1の核酸を結合させた核酸複合体と、第1の核酸と相補的に結合し得る第2の核酸を固相に結合させた核酸固定固相とを用い、前記核酸結合体と前記試料中の測定対象物とを反応させて少なくとも測定対象物と第1の核酸を含んだ複合体を形成させ、該複合体中の第1の核酸を前記第2の核酸と相補結合させて少なくとも測定対象物質と第1の核酸を含んだ複合体を前記核酸の相補結合を介して固相上に不動化させ、固相を試料と分離した後、第1の核酸と第2の核酸の相補結合が解離する条件下で測定対象物を含む複合体を固相から乖離させて固相を除いた測定液を得ることを特徴とする生体成分のアフィニティ分析法を提供するものである。

【0012】また前記請求項3の発明の特徴は、前記の第1の核酸と第2の核酸の相補結合を解く解離を、該相補結合の安定性に関与する因子である塩濃度、温度の少なくともいずれかを制御することにより与えるようにしたところにある。

【0013】固相から乖離した（離れた）複合体に含まれる測定対象物質の定量測定は従来既知の方法を用いて行なうことができる。例えば、測定対象物に対して親和性を有する物質として抗体を用い、第一抗体に第1の核酸を結合させた核酸結合体に酵素（あるいはこれに代えて放射性同位元素、蛍光物質、発光物質などであってもよい）を標識物として含ませておく、あるいは酵素等の標識物質を結合した第二抗体と前記核酸結合体の第一抗体とをそれぞれ測定対象物質と免疫反応させ、結合して形成された複合体中に含まれる標識酵素の反応により基質に現われる蛍光強度の変化を測定するなどの方法を例示することができる。

【0014】

【作用】以下本発明を詳細に説明する。

【0015】本発明に使用される測定対象物と親和性を有する物質とは、抗原－抗体反応における抗原と抗体、リガンド・レセプター反応におけるホルモンあるいはサイトカインなどとそれに対応するレセプター、酵素反応における酵素と基質あるいは補酵素などのある特異的な相互作用でもって互いに結合することのできる個々の分子を意味する。生体外での複合体形成法にあたっては、関与する成分が生体内に通常存在する場合にこれらの成分同士の結合が実際行われる生理的条件下にできる限り近づけて行うのが好ましいが、本発明の実施に先立って予備的に実験を行い、複合体の形成を確認しておけば問題はない。

【0016】本発明に使用される核酸は、互いに相補的な配列からなる２種類の１本鎖の核酸であれば使用可能であり、特に限定されるものではないが長さとして５～５０塩基程度の範囲より適宜選定することができる。

【0017】前記の核酸結合体は、例えば核酸の５′末端にアミノ基を導入し、これを被結合体に存在するアミノ基やチオール基など、あるいは被結合体に導入されたアミノ基やチオール基などに架橋剤を用いて結合させるといった方法で調製することができる。架橋剤としては、Ｎ－サクシニミジル－４－マレイミドブチレート、Ｎ－サクシニミジル－６－マレイミドヘキサノエート、Ｎ－サクシニミジル－３－（２′－ピリジルジチオ）プロピオネートなどが例示できる。

【0018】本発明において用いられる固相としては、例えば、スチレン、エチレングリコール、アクリル酸、メタクリル酸などのポリマー系またはコポリマー系の材料を用いて形成した固相、またはこれらにトシル基、トレシル基、エポキシ基などの反応性官能基を導入した固相、デンプン、デキストラン、セルロース、アガロースなどの多糖類のハロゲン化シアン活性化物あるいはメタ過ヨウ素酸ナトリウム活性化物などの固相等を挙げることができる。固相の形状としては、プレート状、ビーズ状、ゲル状、チューブ状などが例示される。

【0019】第２の核酸としては未修飾の核酸または第１の核酸と同様に例えば５′末端にアミノ基などを導入したものが使用できる。そして第２の核酸の固相への結合方法としては、物理的に吸着させる方法や固相表面に導入された反応性官能基と核酸の末端に導入されたアミノ基などの官能基とを共有結合させる方法などがある。

【0020】測定対象物と核酸結合体とを含む複合体を、固相に結合された核酸との相補結合によって固相に不動化した状態に形成させる方法としては、

（１）核酸結合体を固相に結合された核酸との相補結合

によってまず不動化し、次ぎに測定対象物を含む成分を、該核酸結合体に含まれる物質（例えば抗体）との親和性によって複合体を形成させて固相に不動化させる方法

（2）測定対象物と核酸結合体に含まれる物質（例えば抗体）とを液相反応させることにより複合体を形成させ、次ぎに該複合体を固相に結合された核酸との相補結合によって不動化させる方法

などが挙げられる。

【0021】相補結合した核酸を介して固相に不動化された測定対象物を含む複合体を、該固相から選択的に乖離させる方法としては、核酸の相補結合の安定性に関与する因子、例えば塩濃度、温度、変性剤などを調節する方法を挙げることができる。その中でも塩濃度を調節する方法、具体的には該相補結合に至適な塩濃度とされている溶液を、この相補結合を解離して核酸が１本鎖になる塩濃度に低下させる調節方法によって、容易に達成できる。塩の種類としては、リチウム塩、ナトリウム塩、カリウム塩などが例示される。後述する実施例に示すように生体成分の反応温度に通常よく用いられる２５～３７℃程度において核酸を相補結合させる場合には塩濃度を１M程度以上、１本鎖に解離させる場合には塩濃度を該塩濃度より低下させ、最も好ましくは０M程度とすることにより容易に目的が達成できる。生理的塩濃度において核酸を相補結合させる場合には温度を４～２０℃程度、一本鎖に解離させる場合には温度を４０～６０℃程度のそれぞれの範囲より適宜選択することにより容易に目的が達成できる。本発明の方法は、上述のように相補結合および解離反応を塩濃度あるいは温度のいずれか一方を調節することによって行なっても目的は達成できるが、両者を同時に調節することによって行なう方が、効率よく反応させることができるという面において好ましい。相補結合反応および解離反応における塩濃度条件や温度条件は、用いる核酸塩基の数、組成により異なるが、分析するに当たっては予備的に実験し、最適条件を求めておくのが好ましい場合が多い。

【0022】

【発明の効果】本発明の核酸の相補結合を利用した生体成分のアフィニティ分析法によれば以下の効果が得られる。

【0023】（1）固相から測定対象物を含む成分を選択的に乖離させることにより、固相に非特異的に吸着した標識物と分離して測定対象物を測定できるため、バックグランドが低減でき、その結果、高感度に生体成分を分析することができる。

【0024】（2）固相から測定対象物を含む成分を選択的に乖離させる方法として、塩濃度を変化させることにより行なえば迅速な測定が達成でき、測定時間の短縮化が図れる。

【0025】

【実施例】以下さらに実施例により本発明を説明する。

【0026】実施例1

Tresyl-NPRゲル（２．５μm；東ソー製）に５’末端アミノ化オリゴdＴ３５ｍｅｒを結合させ、オリゴdＴ３５ｍｅｒ固定化ゲルを得た。また、抗ヒトインスリンモノクローナル第一抗体をＦａｂ’化し、そのヒンジ部分に５’末端アミノ化オリゴdＡ１５ｍｅｒをN－サクシニミジル－４－マレイミドブチレートを介して結合させ、オリゴdＡ１５ｍｅｒ核酸結合体を得た。

【0027】この第一抗体を含む核酸結合体を１M ＮａＣｌ存在下、該オリゴdＴ３５ｍｅｒ固定化ゲルとハイブリダイズさせて抗体固定化ゲルを調製した。該抗体固定化ゲルに０．５M ＮａＣｌ存在下、アルカリフォスファターゼ標識抗ヒトインスリンモノクローナル第二抗体、次ぎにヒトインスリンを添加し、室温にて３０分間反応させた。０．５M ＮａＣｌを含む洗浄液を用いて２回洗浄を行った後、免疫複合体形成ゲルに０．０２％牛血清アルブミンを含むリン酸緩衝液（０MＮａＣｌ）を添加して４８℃，１０分間、固相からの乖離反応を行った。

【0028】得られた免疫複合体画分に４－メチルウンベリフェリルリン酸を添加し、３７℃、２０秒間反応させたときの活性レートを蛍光測定した。得られた検量線を第１図に示した。

【0029】実施例2

Tresyl-NPRゲル（２．５μm；東ソー製）に５’末端アミノ化オリゴdＴ３５ｍｅｒを結合させ、オリゴdＴ３５ｍｅｒ固定化ゲルを得た。また、抗ヒトインスリンモノクローナル第一抗体をＦａｂ’化し、そのヒンジ部分に５’末端アミノ化オリゴdＡ１５ｍｅｒをN－（ε－マレイミドカプロリロキシ）サクシイミドを介して結合させ、オリゴdＡ１５ｍｅｒ結合第一抗体として核酸結合体を得た。

【0030】この第一抗体を含む核酸結合体を１M ＮａＣｌ共存下、該オリゴdＴ３５ｍｅｒ固定化ゲルとハイブリダイズさせて抗体固定化ゲルを調製した。該抗体固定化ゲルに０．５M ＮａＣｌ共存下、アルカリフォスファターゼ標識抗ヒトインスリンモノクローナル第二抗体、次にヒトインスリンを添加し、室温にて１０分間反応させた。０．５M ＮａＣｌを含む洗浄液を用いて４回洗浄を行った後、免疫複合体形成ゲルに５３℃に予備加熱した０．０２％牛血清アルブミンを含むトリス塩酸緩衝液（０M ＮａＣｌ）を添加して５秒間ミキサーで撹拌し、固相からの乖離反応を行った。

【0031】得られた免疫複合体画分に４－メチルウンベリフェリルリン酸を添加し、３７℃、１００秒間反応させたときの活性レートを蛍光測定した。得られた検量線を図３に示した。

【0032】また２SD法による検出下限界値を求めたところ、測定試料として２５μｌを用いた場合、５ｐｇ

／ml（125fg＝20amol／assay）であった。得られた結果を図4に示した。なおこの図4のmean＋2SD（n＝10）とあるのは、0濃度の試料を10回測定した平均値（プロットしている箇所）＋2SD（SD：標準偏差）のことをいい、エラーバーの上限値から検量線に向かって水平線を引き、交差した点よりインスリンの濃度を読み取るという2SD法により検出下限値を求めたことを示している。

【0033】以上の実施例のように、本発明によれば、固相に固定化した核酸と核酸結合体の核酸との相補結合を利用して、測定対象物であるヒトインスリン及び測定用標識である酵素を含む免疫複合体を固相に不動化し、その後、核酸の相補結合を解いて該免疫複合体を回収して高密度の測定を行うことができる。なお本発明は前記実施例に限定されるものではなく、本発明の要旨を変更しない範囲で種々異なった態様で行うことができる。

【0034】例えば、前記調製のオリゴdA15mer標識抗ヒトインスリンモノクローナル第一抗体にアルカリフォスファターゼ標識抗ヒトインスリンモノクローナル第二抗体およびヒトインスリンを添加して液相にて抗原抗体反応を行い、生成した免疫複合体をオリゴdT35mer固定化ゲルに捕捉させ、ゲルを洗浄したのち固相からの乖離反応を行って、活性レートを測定する方法によっても、良好な結果を得ることができた。

【0035】前記実施例は塩濃度と温度の両方の因子または温度因子を調節することによって目的を達成した例であるが、以下の実験結果によると一定温度下において塩濃度を変化させる方法あるいは一定塩濃度下において温度を変化させる方法によっても目的を達成できることは明らかである。

【0036】すなわち、実施例1の方法にて調製したオリゴdT35mer固定化ゲルおよびオリゴdA15mer結合第一抗体を用い、2M　NaCl存在下においてハイブリダイズさせ、抗体固定化ゲルを調製した。つぎにこの第一抗体の固相からの乖離反応を25，37，48℃のそれぞれにおいてNaCl濃度を2．0から0Mまで段階的に減少させた条件にて行い、遠心分離後、乖離して上清中に存在する第一抗体量をHPLCにて定量した。得られた第一抗体の固相からの乖離割合（％）とNaCl濃度（M）の関係を図2に示した。48℃において1M　NaCl存在下で相補結合させ、次に0M　NaClにすれば100％の乖離が認められた。また0．25M　NaClにおいて25℃で相補結合させ、次に48℃にすれば約70％の乖離が認められた。実施例2にも示したが、以上のように相補結合反応および解離反応を濃度あるいは温度のいずれか一方のみを調節することによっても目的は容易に達成できる。

【0037】なお、実施例2において、インスリン陰性血清および健常人の空腹時血清を用いて、同時再現性の評価を実施した結果、平均濃度42．6pg／ml（約1μU／ml）の健常人空腹時血清が変動係数8．4％と良好に測定できた。得られた結果を図5に示した。

【図面の簡単な説明】

【図1】実施例1により得られた検量線を示した図。

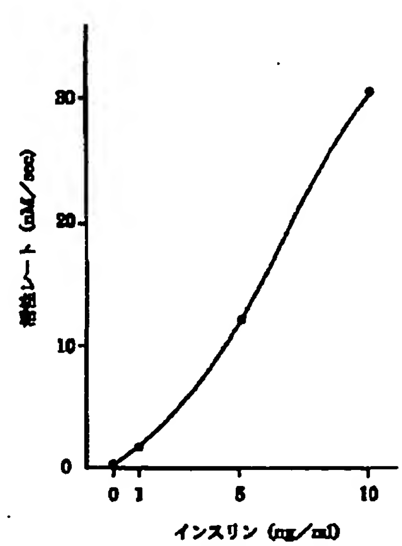
【図2】実施例1ににおける、オリゴdA15mer結合第一抗体の25℃，37℃，48℃での乖離割合（％）とNaCl濃度（M）の関係を示した図。

【図3】実施例2により得られた検量線を示した図。

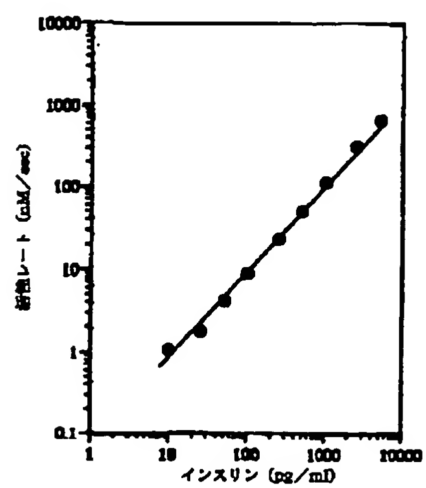
【図4】実施例2における検出限界値を示した図。

【図5】実施例2における、インスリン陰性血清および健常人の空腹時血清を用いて、同時再現性を評価した結果を示す図。

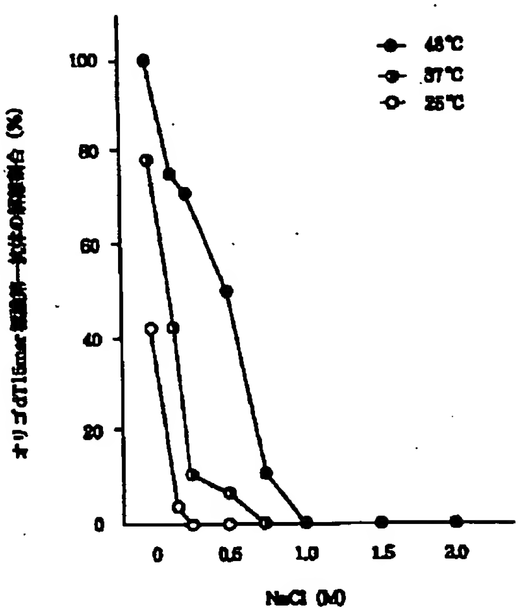
【図1】



【図3】

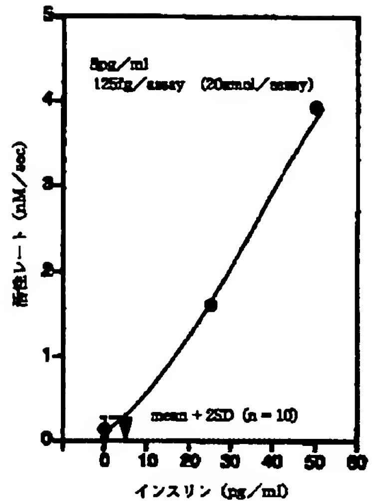


【図2】



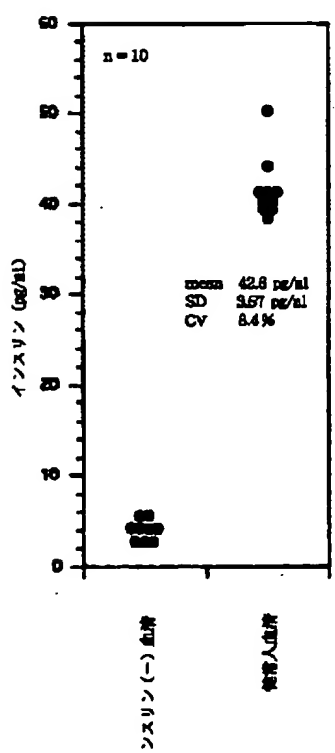
45℃
37℃
25℃
NaCl (M)

【図4】



5pg/ml
125fg/assay (20amol/assay)
mean + 2SD (n = 10)
インスリン (pg/ml)

【図5】



n = 10
mean 42.8 pg/ml
SD 3.57 pg/ml
CV 8.4%
インスリン (pg/ml)
インスリン(ー)血清
健常人血清

フロントページの続き

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(54) Title of the Invention: Method of affinity analysis for biological components

(57)

Abstract

Purpose:

To provide a highly sensitive method of analysis in which an immuno-complex which contains the substance to be measured and a label is separated from a solid phase quickly, easily and selectively, and with which it is possible to reduce the background originating from non-specifically adsorbed fractions.

Constitution:

Using a nucleic acid bound unit, where a nucleic acid has been bound to a substance which has an affinity for the substance to be measured, and a solid phase to which a nucleic acid complementary with the said nucleic acid has been bound, in a state in which an immuno-complex which contains the substance to be measured, the said nucleic acid bound unit and enzyme is immobilised by the complementary bonding of the nucleic acid which has been bound to the solid phase and the said nucleic acid bound unit is formed and then, by adjusting a factor which imparts stability to the said complementary bonding, the immuno-complex which contains the substance to be measured and the enzyme is separated selectively from the said solid phase.

Scope of the Patent Claims

[Claim 1]

A method of affinity analysis for biological components in which a substance to be measured in a biological sample is measured after it has been captured selectively on a solid phase and separated from other substances which are also present in the sample, characterised in that, using a nucleic acid bound unit, where a first nucleic acid has been bound to a substance which has an affinity for the substance to be measured, and a nucleic acid-affixed solid phase where a second nucleic acid which can undergo complementary bonding with the first nucleic acid has been bound to a solid phase, the substance which has an affinity for the substance to be measured which is included in the aforementioned nucleic acid bound unit and the aforementioned substance to be measured in the sample are reacted under conditions where complementary bonding occurs between the first and second nucleic acids and a complex which contains at least the substance to be measured and the first nucleic acid is immobilised on the solid phase by the complementary bonding of the aforementioned nucleic acids and, after separating the solid phase from the sample, a complex which contains the substance to be measured is released from the solid phase under conditions where the complementary bonding between the first and second nucleic acids is dissociated and a liquid for measurement purposes from which the solid phase has been removed is obtained.

[Claim 2]

A method of affinity analysis for biological components wherein a substance to be measured in a biological sample is measured after it has been

captured selectively on a solid phase and separated from other substances which are also present in the sample, characterised in that, using a nucleic acid bound unit, where a first nucleic acid has been bound to a substance which has an affinity for the substance to be measured, and a nucleic acid-affixed solid phase where a second nucleic acid which can undergo complementary bonding with the first nucleic acid has been bound to a solid phase, the substance to be measured in the aforementioned sample and the aforementioned nucleic acid bound unit are reacted and a complex which contains at least the substance to be measured and the first nucleic acid is formed, the first nucleic acid in the said complex is subjected to complementary bonding with the aforementioned second nucleic acid and a complex which contains at least the substance to be measured and the first nucleic acid is immobilised on the solid phase by the complementary bonding of the aforementioned nucleic acids and, after separating the solid phase from the sample, the complex which contains the substance to be measured is released from the solid phase under conditions where the complementary bonding between the first and second nucleic acids is dissociated and a liquid for measurement purposes from which the solid phase has been removed is obtained.

[Claim 3]

A method of affinity analysis, according to claim 1 or claim 2, characterised in that the conditions under which the complementary bonding of the first nucleic acid and the second nucleic acid is dissociated are achieved by adjusting at least either the salt concentration or the temperature which are

factors which contribute to the stability of the said complementary bonding.

[Claim 4]

A method of affinity analysis, according to any of claims 1 to 3, characterised in that the complex which contains the substance to be measured and the first nucleic acid includes a labelling substance for measurement purposes.

Detailed Description of the Invention

[0001]

Industrial Field of Application

The invention concerns a method with which biological components are analysed using interactions therewith.

[0002]

Prior Art

Immunoassay in which an antibody corresponding to the component to be measured is used and receptor-assay where a receptor is used, for example, are known methods for the measurement of trace components in biological samples with high sensitivity and specificity.

[0003]

The general principal of measurement in high sensitivity immunoassay is described below, and here an antibody for the component (antigen) to be measured is first fixed to a support, such as a tube, beads or a plate for example, (to form a solid phase) and then the sample is added to this and the antigen is captured by the solid phase antibody. The other substances present in the sample are removed and then an excess of a labelled antibody (an antibody to which a labelling substance has been bound) which recognises a different part of the antigen from the solid phase antibody is

added and bound with the antigen which has been captured on the solid phase. Radioactive isotopes, enzymes, fluorescent substances and luminescent substances, for example, can be used for the labelling substance of the aforementioned labelled antibody. The bound labelled antibody is proportional to the amount of antigen and so the amount of antigen can be determined by determining the amount of labelling substance on the solid phase after removing the unreacted labelled antibody by washing.

[0004]

However, with the aforementioned general principle of measurement, the labelled antibody is added in excess and so the labelled antibody which is adsorbed non-specifically on the surface of the solid phase is not removed completely by washing and is left behind, causing the background in the measuring system to rise. The problem is that it is impossible to achieve high sensitivity.

[0005]

That is to say, with the conventional method as described earlier, a labelled antibody is added in excess to a reaction liquid which contains the solid phase support and, after washing, the labelled substance complex which has been formed on the solid phase by the antigen-antibody reaction is detected directly without being separated from the said solid phase and so the labelled antibody which has been adsorbed non-specifically on the surface of the solid phase is detected at the same time and, as a result, there is a rise in the background level.

[0006]

Hence, attempts have been made to determine the amount of labelling substance after releasing the

immuno-complex which has formed on the solid phase from the solid phase and, for example, a method in which the bonds have been broken using a reducing agent after the immuno-complex has been captured on a gel by means of sulphide bonds, the immuno-complex being eluted and the labelling substance being determined has been reported. (Rinsho Kensa, 28, 909-916, 1984). However, with this method the reducing agent which is used to release the complex from the solid phase denatures the antibody molecules and may also release the labelled antibody which is non-specifically adsorbed and the labelled substance may be separated, and it is not possible to selectively release the immuno-complex which is the subject for measurement.

[0007]

Furthermore, a method in which the immuno-complex is captured on a solid phase via a nucleic acid, the said nucleic acid being broken using a limiting enzyme and the amount of labelling substance being determined has also been reported (Imai et al., Japanese Unexamined Patent Application Laid Open H4-204379). However, in this case a method of release involving the use of an enzyme is employed and so the process takes a long period of time. This is disadvantageous in terms of shortening the overall measurement time and reducing the running costs.

[0008]

Problems to be Resolved by the Invention

The aim of the invention is to provide a highly sensitive analytical procedure whereby an immuno-complex which includes the substance to be measured and a label can be separated from a solid phase both quickly and selectively, thereby enabling the effect of a raised background level originating from a fraction

which has been adsorbed non-specifically on the solid phase to be reduced.

[0009]

Moreover, another aim of the invention is to provide a simple adjustment as the means of separating the substance to be measured selectively from the solid phase.

[0010]

Means of Resolving These Problems

Thus, the invention of claim 1 provides a method of affinity analysis for biological components wherein a substance to be measured in a biological sample is measured after it has been captured selectively on a solid phase and separated from other substances which are also present in the sample which is characterised in that, using a nucleic acid bound unit, where a first nucleic acid has been bound to a substance which has an affinity for the substance to be measured, and a nucleic acid-affixed solid phase where a second nucleic acid which can undergo complementary bonding with the first nucleic acid has been bound to a solid phase, the substance which has an affinity for the substance to be measured which is included in the aforementioned nucleic acid bound unit and the aforementioned substance to be measured in the sample are reacted under conditions where complementary bonding occurs between the first and second nucleic acids and a complex which contains at least the substance to be measured and the first nucleic acid is immobilised on the solid phase by the complementary bonding of the aforementioned nucleic acids and, after separating the solid phase from the sample, the complex which contains the substance to be measured is released from the solid phase under conditions where the complementary bonding

between the first and second nucleic acids is dissociated and a liquid for measurement purposes from which the solid phase has been removed is obtained.

[0011]

Furthermore, the distinguishing feature of the invention of claim 2 is a method of affinity analysis for biological components wherein a substance to be measured in a biological sample is measured after it has been captured selectively on a solid phase and separated from other substances which are also present in the sample which is characterised in that, using a nucleic acid bound unit, where a first nucleic acid has been bound to a substance which has an affinity for the substance to be measured, and a nucleic acid-affixed solid phase where a second nucleic acid which can undergo complementary bonding with the first nucleic acid has been bound to a solid phase, the substance to be measured in the aforementioned sample and the aforementioned nucleic acid bound unit are reacted and a complex which contains at least the substance to be measured and the first nucleic acid is formed, the first nucleic acid in the said complex is subjected to complementary bonding with the aforementioned second nucleic acid and a complex which contains at least the substance to be measured and the first nucleic acid is immobilised on the solid phase by the complementary bonding of the aforementioned nucleic acids and, after separating the solid phase from the sample, the complex which contains the substance to be measured is released from the solid phase under conditions where the complementary bonding between the first and second nucleic acids is dissociated and a liquid for measurement purposes from which the solid phase has been removed is obtained.

[0012]

Moreover, the distinguishing feature of the invention of claim 3 is that the dissociation of the complementary bonding of the first nucleic acid and the second nucleic acid is achieved by adjusting at least either the salt concentration or the temperature which are factors which contribute to the stability of the said complementary bonding.

[0013]

The quantitative measurement of the substance to be measured included in the complex which has been separated (released) from the solid phase can be carried out using conventional methods. For example, use can be made of the methods where, using an antibody as a substance which has an affinity for the substance to be measured, an enzyme (or alternatively a radioactive isotope, a fluorescent substance or a luminescent substance) is included as a labelling substance in a nucleic acid bound unit where the first nucleic acid has been bound with the first antibody, or where the aforementioned nucleic acid bound unit and a second antibody to which a labelling substance such as an enzyme has been bound are both made to undergo an immuno-reaction with the substance to be measured. The change in the fluorescence intensity seen in a substrate as a result of the reaction of the labelling enzyme which is bound to the complex which is formed by the bonding, for example, is measured.

[0014]

Action

The invention is described in more detail below.

[0015]

The substance which has an affinity with the substance to be measured which can be used in the

invention is a substance which has an certain specific interaction like that of the antigen and the antibody in an antigen-antibody reaction, the receptor corresponding to the hormone or cytokine in a ligand-receptor reaction, or the enzyme and substrate or coenzyme in an enzyme reaction, signifying that individual molecules can be bound together. In the method of forming a complex in vitro it is desirable that in those cases where the components which participate are generally present in vivo, the bonding together of these components should be carried out under conditions as close as possible to the physiological conditions in which it would occur naturally. There is, however, no problem in the execution of this invention if preliminary experiments are carried out beforehand and the formation of the complex is confirmed.

[0016]

Provided that it is a nucleic acid which has two types of single chain comprising mutually complementary base sequences, any nucleic acid can be used for the nucleic acid which is used in this invention and, although no particular limitation is imposed, those with a length in the range from some 5 to 50 bases can be selected appropriately.

[0017]

The aforementioned nucleic acid bound unit can be prepared, for example, using a method in which an amino group is introduced on the 5′-end of the nucleic acid and this is bound to an amino group or thiol group, for example, which is present in the unit to be bound, or with an amino group or thiol group which has been introduced into the unit to be bound, using a crosslinking agent. Examples of such crosslinking

agents include N-succinimidyl-4-maleimidobutyrate, N-succinimidyl-6-maleimidohexanoate and N-succinimidyl-3-(2'-pyridyldithio)propionate.

[0018]

The solid phase which is used in the invention may be, for example, a solid phase which has been formed using a polymer or copolymer based material such as styrene, ethylene glycol, acrylic acid, methacrylic acid or the like, or a solid phase of this type into which reactive functional groups such as tosyl groups, tresyl groups, epoxy groups and the like have been introduced, or a solid phase such as a cyanohalide activated or sodium metaperiodate activated polysaccharide such as starch, dextran, cellulose, agarose or the like. The solid phase may have, for example, a plate-like, bead-like, gel-like or tube-like form.

[0019]

Unadorned nucleic acids and nucleic acids which have an amino group introduced on the 5'-end, for example, like the first nucleic acid, can be used for the second nucleic acid. The methods involving physical adsorption and the methods involving covalent bonding between reactive functional groups which have been introduced onto the surface of the solid phase and functional groups such as amino groups which have been introduced on the end of the nucleic acid, for example, can be used for binding the second nucleic acid to the solid phase.

[0020]

The methods indicated below can be cited as methods whereby the complex which includes the substance to be measured and the nucleic acid bound unit can be formed in a state where they are

immobilised on a solid phase by complementary bonding with the nucleic acid which has been bound to the solid phase:

(1) The method in which the nucleic acid bound unit is first immobilised by complementary bonding with the nucleic acid which has been bound to the solid phase and then the component which contains the substance to be measured is immobilised on the solid phase by forming a complex as a result of an affinity with the substance (for example an antibody) which is included in the said nucleic acid bound unit.

(2) The method in which a complex is formed by means of a liquid phase reaction of the substance to be measured and the substance (for example an antibody) which is included in the nucleic acid bound unit and then the said complex is immobilised by means of complementary bonding with the nucleic acid which has been bound to the solid phase.

[0021]

The methods in which a factor which contributes to the stability of the nucleic acid complementary bonding, for example the salt concentration, the temperature or a denaturant, for example, is adjusted can be cited as methods whereby the complex which contains the substance to be measured which has been immobilised on the solid phase via the complementary bound nucleic acids can be selectively released from the said solid phase. From among these methods, the effect is most easily achieved by using a method in which the salt concentration is adjusted, and in more practical terms by means of a method of adjustment in which a solution which has been set to an appropriate salt concentration for the said complementary bonding has the salt concentration reduced to a level at which

the complementary bonding dissociates and the nucleic acids form single chains. Lithium salts, sodium salts and potassium salts, for example, can be used for the salt. As shown in the illustrative examples described hereinafter, a situation where the nucleic acids are complementary bonded at a temperature of from 25 to 37°C, as generally used for the reaction temperature of biological components, can be achieved easily with a salt concentration above about 1M, and a situation where there is dissociation into single chains can be achieved easily by reducing the salt concentration below the said salt concentration and, most desirably, by setting the salt concentration to about 0M. A situation where the nucleic acids are complementary bonded at the physiological saline concentration can be achieved easily by selecting a temperature in the range from 4 to 20°C, and a situation where there is dissociation into single chains can be achieved easily by selecting a temperature within the range from 40 to 60°C. In the method of this invention the complementary bonding and its dissociation can be achieved easily by adjusting either the salt concentration or the temperature as described above, but adjusting both of these factors at the same time is preferred in that under these circumstances the reactions can be carried out more efficiently. The salt concentration conditions and temperature conditions for the complementary bonding reaction and the dissociation reaction differ according to the number and composition of nucleic acid bases involved, and in most cases it is desirable that the optimum conditions for analysis should be determined by carrying out preliminary experiments.

[0022]

Effect of the Invention

The following effects are obtained with the method for the affinity analysis for biological components of this invention in which the complementary bonding of nucleic acids is used.

[0023]

(1) It is possible to separate the labelling substance which has been adsorbed non-specifically on the solid phase by separating the component which contains the substance to be measured selectively from the solid phase and to measure the substance to be measured. In this way the background can be reduced and, as a result, it is possible to analyse biological components with greater sensitivity.

[0024]

(2) If changing the salt concentration is used as the method for separating the component which contains the substance to be measured selectively from the solid phase then the measurement can be achieved quickly and the measurement time is shortened.

[0025]

Illustrative Examples

[0026] **Example 1**

5'-Terminal-amino-oligo-dT35-mer was bound to tresyl-NPR gel (2.5 μm: manufacture by Toso) to obtain a gel to which oligo-dT35-mer had been fixed. Furthermore, a first anti-human insulin monoclonal antibody was Fab'-ised and 5'-terminal-amino-oligo-dA15-mer was bound by means of N-succinimidyl-4-maleimidobutylate to the hinge-part of this antibody and an oligo-dA15-mer nucleic acid bound unit was obtained.

[0027]

A gel to which antibody had been fixed was prepared by hybridising the nucleic acid bound unit which contained this first antibody with the said gel to which the oligo-dT35-mer had been fixed in the presence of 1M NaCl. An second alkali phosphatase labelled anti-human insulin monoclonal antibody and then human insulin were added in the presence of 0.5M NaCl to the said gel to which the antibody had been fixed and reacted for 30 minutes at room temperature. After washing twice using a washing solution which contained 0.5M NaCl, phosphoric acid buffer solution (0M NaCl) which contained 0.02% bovine serum albumin was added to the gel on which the immuno-complex had been formed and a separation from the solid phase reaction was carried out at 48°C for 10 minutes.

[0028]

4-Methylumbelliferyl phosphate was added to the immuno-complex fraction obtained and the activity rate on reacting for 20 seconds at 37°C was measured using the fluorescence. The calibration curve obtained is shown in figure 1.

[0029]

Example 2

5'-Terminal-amino-oligo-dT35-mer was bound to tresyl-NPR gel (2.5 μm: manufacture by Toso) to obtain a gel to which oligo-dT35-mer had been fixed. Furthermore, a first anti-human insulin monoclonal antibody was Fab'-ised and 5'-terminal-amino-oligo-dA15-mer was bound by means of N-(ε-maleimido-caprolyloxy)succinimide to the hinge-part of this antibody and a nucleic acid bound unit was obtained as an oligo-dA15-mer bound first antibody.

[0030]

A gel to which antibody had been fixed was prepared by hybridising this nucleic acid bound unit which contained the fist antibody with the said gel to which the oligo-dT35-mer had been fixed in the presence of 1M NaCl. A second alkali phosphatase labelled anti-human insulin monoclonal antibody and then human insulin were added in the presence of 0.5M NaCl to the said gel to which the antibody had been fixed and reacted for 10 minutes at room temperature. After washing four times using a washing solution which contained 0.5M NaCl, Tris hydrochloric acid buffer solution (0M NaCl) which contained 0.02% bovine serum albumin which had been pre-heated to 53°C was added to the gel on which the immuno-complex had been formed, the mixture was stirred for 5 seconds in a mixer and a separation from the solid phase reaction was carried out.

[0031]

4-Methylumbelliferyl phosphate was added to the immuno-complex fraction obtained and the activity rate on reacting for 100 seconds at 37°C was measured using the fluorescence. The calibration curve obtained is shown in figure 3.

[0032]

Furthermore, the lowest detection limit was obtained using the 2 SD method, and when 25 μl sample was used the value was 5 pg/ml (125 fg = 20 amol/assay). The results obtained are shown in figure 4. Moreover, the mean + 2 SD (n =10) value shown in figure 4 is the average value (plotted position) on measuring a sample of zero concentration ten times + 2 SD (SD: standard deviation), and the minimum limit for detection with the 2 SD method shown was determined by drawing a horizontal line toward the

calibration curve from the upper limiting value of the error bar and reading off the concentration of insulin at the point of intersection.

[0033]

As indicated in the examples described above, by means of this invention it is possible to immobilise an immuno-complex which contains an enzyme for measurement labelling purposes and human insulin which is the substance to be measured on a solid phase using complementary bonding between a nucleic acid which has been fixed on a solid phase and the nucleic acid of the nucleic acid bound unit, to break down the nucleic acid complementary bonding and recover the said immuno-complex, and to carry out measurement with a high degree of accuracy. Moreover, the invention is not limited to the aforementioned examples and it can be executed with various modifications without changing the essential nature of the invention.

[0034]

For example, good results can be obtained with a method in which the second alkali phosphatase labelled anti-human insulin monoclonal antibody and the human insulin are added to the aforementioned oligo-dA15-mer labelled first anti-human insulin monoclonal antibody preparation, and an antigen-antibody reaction is carried out in the liquid phase. The immuno-complex which is formed is trapped on a gel to which oligo-dT35-mer has been fixed and, after washing the gel, a separation from the solid phase reaction is carried out and the activity rate measured.

[0035]

In the aforementioned examples the objective was realised by adjusting both the salt concentration and temperature factors or the temperature factor but,

according to the experimental results outlined below, it is clear that the objective can be realised with a method in which the salt concentration is changed at a fixed temperature or a method in which the temperature is changed at a fixed salt concentration.

[0036]

Thus, using the gel to which the oligo-dT35-mer had been fixed and the oligo-dA15-mer bound first antibody prepared in the method of example 1, hybridisation was carried out in the presence of 2M NaCl to prepare a gel to which the antibody had been fixed. Then, the separation reaction of the first antibody from the solid phase was carried out under conditions where the NaCl concentration was reduced in stages from 2.0 to 0M at temperatures of 25, 37 and 48°C and, after centrifugal separation, the amount of the first antibody which had been separated and was present in the supernatant liquid was determined using HPLC. The relationship between the proportion of the first antibody released from the solid phase and the NaCl concentration (M) obtained is shown in figure 2. It was confirmed that at 48°C there was 100% separation if complementary bonding was carried out in the presence of 1M NaCl and then the system was set to 0M NaCl. Furthermore, about 70% separation was confirmed on complementary bonding at 25°C in 0.25M NaCl if the temperature was then set to 48°C. Although shown in example 2 as well, the object of the invention can be realised easily by adjusting either the concentration or the temperature of the complementary bonding reaction and the separating reaction in the ways described above.

[0037]

Moreover, insulin negative serum and serum from a healthy person on fasting were used in example 2 and reproducibility was evaluated at the same time, and the serum from a healthy person after fasting with an average concentration of 42.6 pg/ml (about 1 μU/ml) could be measured very well with a coefficient of variation of 8.4%. The results obtained are shown in figure 5.

Brief Explanation of the Drawings

Figure 1 is a drawing which shows the calibration curve obtained in example 1.

Figure 2 is a drawing which shows the relationship between the NaCl concentration (M) and the proportion of oligo-dA15-mer bound first antibody separated at 25°C, 37°C and 48°C in example 1.

Figure 3 is a drawing which shows the calibration curve obtained in example 2.

Figure 4 is a drawing which shows the limit of detection in example 2.

Figure 5 is a drawing which shows the results obtained on evaluating the reproducibility using insulin negative serum and serum from a healthy person after fasting.

Figure 1

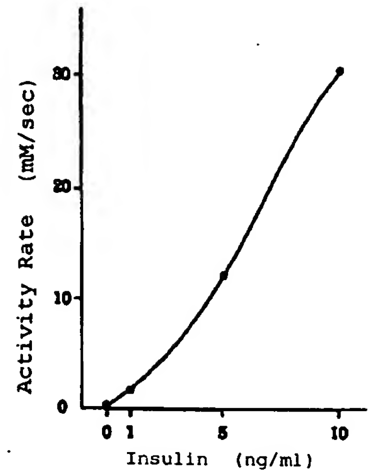


Figure 2

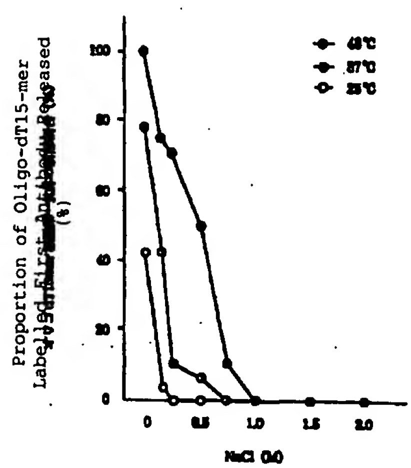


Figure 3

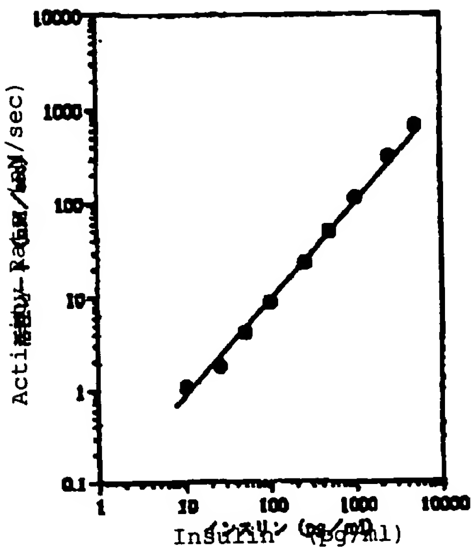


Figure 4

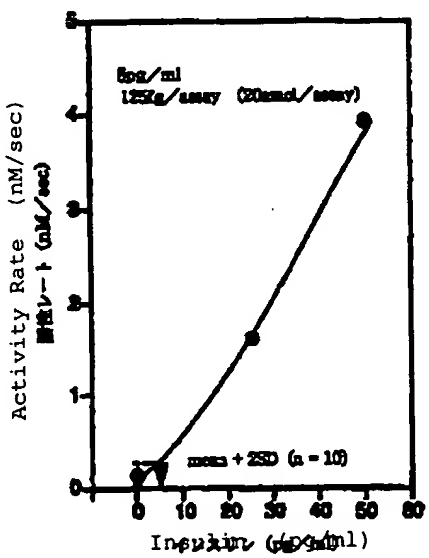
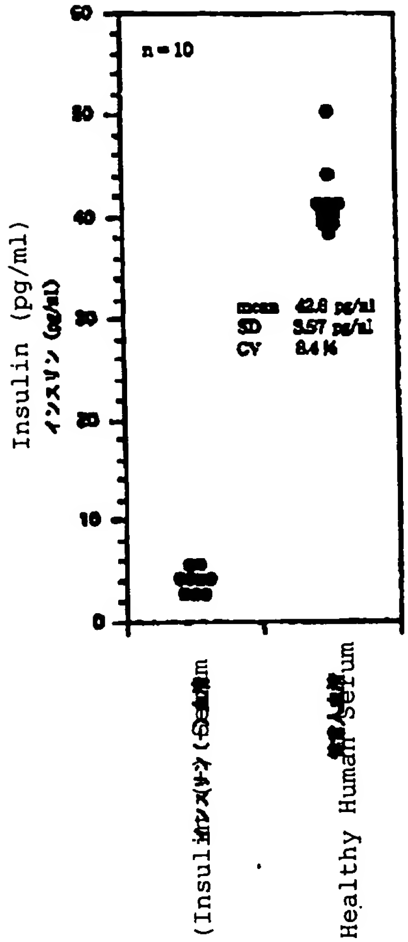


Figure 5


n = 10
mean 42.6 pg/ml
SD 3.57 pg/ml
CV 8.4 %
Insulin (pg/ml)
Healthy Human Serum